α_2-Antiplasmin Supplementation Inhibits Tissue Plasminogen Activator–induced Fibrinogenolysis and Bleeding with Little Effect on Thrombolysis

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Abstract

Tissue plasminogen activator (t-PA) causes fibrinogen proteolysis when α_2-antiplasmin levels fall, and this may contribute to t-PA–induced hemorrhage. Because clot-bound plasmin is protected from α_2-antiplasmin inhibition, we tested the possibility that α_2-antiplasmin supplementation would block t-PA–induced fibrinogenolysis and bleeding without affecting thrombolysis. When added to human or rabbit plasma, α_2-antiplasmin inhibits t-PA–induced fibrinogenolysis, but has little effect on the lysis of ^{125}I-fibrin clots. To examine its effect in vivo, rabbits with preformed ^{125}I-labeled–jugular vein thrombi were randomized to receive t-PA, t-PA and α_2-antiplasmin, or saline. α_2-Antiplasmin infusion produced a modest decrease in t-PA–induced thrombolysis (from 40.2% to 30.1%, $P = 0.12$), but reduced fibrinogen consumption from 87% to 27% ($P = 0.0001$), and decreased blood loss from standardized ear incisions from 5,594 to 656 μl ($P < 0.0001$). We hypothesize that α_2-antiplasmin limits t-PA–induced hemorrhage by inhibiting fibrinogenolysis and subsequent fragment X formation because (*a*) SDS-PAGE and immunoblot analysis indicate less fragment X formation in α_2-antiplasmin treated animals, and (*b*) when added to a solution of fibrinogen and plasminogen clotted with thrombin in the presence of t-PA, fragment X shortens the lysis time in a concentration-dependent fashion. These findings suggest that fragment X incorporation into hemostatic plugs contributes to t-PA–induced bleeding. By blocking t-PA–mediated fibrinogenolysis, α_2-antiplasmin supplementation may improve the safety of fibrin-specific plasminogen activators. (*J. Clin. Invest.* 1993. 91:1343–1350.) Key words: fibrin degradation • fibrinogenolysis • plasmin inhibitors

Introduction

Despite its affinity for fibrin, tissue-type plasminogen activator (t-PA)[1] administration causes a systemic lytic state with fibrinogen proteolysis (1–4). Although kinetic models predicted that high doses of t-PA would have this effect (5, 6), recent studies suggest an additional mechanism through which even low doses of t-PA can produce fibrinogen breakdown. Thus, we have demonstrated that the degradation products of cross-linked fibrin produced during the thrombolytic process can potentiate t-PA–induced plasminogen activation through their capacity to bind plasminogen and t-PA (7). While the major cause of t-PA–induced bleeding is likely to be lysis of fibrin within the hemostatic plug (8), the induction of the systemic lytic state may also be a contributing factor (3, 4). Therefore, maneuvers that limit the systemic lytic state without impairing the thrombolytic effect of t-PA could be of potential clinical value.

The extent of the systemic lytic state produced by t-PA is critically dependent on the α_2-antiplasmin concentration. Fibrinogen breakdown occurs when the plasma levels of α_2-antiplasmin fall below those necessary to limit plasmin activity. Whereas free plasmin is rapidly complexed and inactivated by α_2-antiplasmin (9–12), once the inhibitor is depleted, unopposed plasmin degrades fibrinogen (13). In contrast to the free enzyme, plasmin bound to fibrin is relatively protected from inhibition by α_2-antiplasmin (10–12). Based on these considerations, we performed experiments in vitro and in vivo to investigate the possibility that α_2-antiplasmin supplementation would block t-PA–induced fibrinogenolysis without inhibiting clot lysis. In addition, we examined the effect of α_2-antiplasmin supplementation on t-PA–induced bleeding.

Methods

Reagents

Predominantly single-chain human recombinant t-PA (Lot K9051A6) was obtained from Genentech, Inc., San Francisco, CA. Plasminogen with an amino-terminal glutamic acid residue (Glu-plasminogen) was purchased from Enzyme Research Laboratories, Inc., South Bend, IN; a mouse monoclonal antibody against human t-PA (PAM-2), and aprotinin were obtained from American Diagnostica, New York, NY. Human plasmin and the synthetic substrate for plasmin, D-norleucyl-L-β-cyclohexylalanyl-L-arginine-paranitroanalide (CBS 33.08), were obtained from Diagnostica Stago, Asnières, France. α_2-Antiplasmin, purified from human plasma as described elsewhere (14), was generously provided by Dr. E. P. Paques, Research Laboratories, Behringwerke AG, Marburg, FRG. On SDS-PAGE, the isolated protein yields a single band of about 70,000 D, and when titrated against a known concentration of plasmin (14, 15), this material inhibits the enzyme in a 1:1 fashion.

The fragment X derivative used in this study was kindly provided by Dr. D. Galanakis, State University of New York, Stony Brook, NY. Isolated from a limited plasmin digest of human fibrinogen by differential ethanol subfractionation (16, 17), it represents an early fragment X species because it is > 90% clottable and has a molecular mass of ~ 270,000 D. Further, comparison of thrombin-induced fibrinopeptide A and B release monitored using reverse-phase HPLC (18) indicates that at least 30% of the amino-terminal Bβ-chains are still intact.

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1. *Abbreviations used in this paper:* DD, D-dimer; (DD)E complex, complex between D-dimer and fibrin fragment E; KIU, kallikrein inhibitor unit; TBS, 0.1 M NaCl buffered to pH 7.4 with 0.05 M Tris-HCl; t-PA, tissue-type plasminogen activator.

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Preparation of ^{125}I-labeled fibrinogen

Fibrinogen was precipitated from barium sulfate adsorbed plasma with 2 M β-alanine (19), and then trace labeled with ^{125}I (20) to a specific activity of 100±5 μCi/mg.

Preparation of ^{125}I-labeled cross-linked fibrin clots

Blood was collected from healthy volunteers or from rabbits into plastic syringes prefilled with 1/10 vol of 3.8% trisodium citrate or with 2 U/ml heparin. After sedimentation of the red cells by centrifugation at 1,700 *g* for 15 min at 4°C, the harvested citrated plasma was supplemented with ^{125}I-labeled fibrinogen (~ 200,000 cpm/ml). 500-μl aliquots were then transferred to polypropylene eppendorf tubes, and labeled cross-linked fibrin clots were formed around wire hooks by the addition of $CaCl_2$ (final concentration, 25 mM). The clots were aged for 60 min at 37°C with constant agitation, and washed three times with 1 ml aliquots of 0.1 M NaCl buffered to pH 7.4 with 0.05 M Tris-HCl (TBS) over the course of 15 min. The washed clots were then counted for radioactivity for 1 min using a Clinigamma counter (LKB Instruments, Gaithersburg, MD).

Clots formed in this fashion are cross-linked because they remain intact after 24 h of incubation in 2% acetic acid. Further, SDS-PAGE analysis under reducing conditions of clots solubilized in SDS (21) demonstrates bands corresponding to the β, γ-γ dimers, and α-polymer chains. Non–cross-linked α or γ chains are not visualized thus indicating virtually complete cross-linking.

Preparation of clot lysates and (DD)E complexes

^{125}I-labeled plasma clots were lysed with t-PA (7), and the clot lysates were then immunodepleted of t-PA using PAM-2, a mouse monoclonal IgG against the kringle-1 region of human t-PA (22), coupled to Sepharose 4B as we have previously described (7). The final material contained < 50 ng of t-PA as measured antigenically using an assay kit from American Diagnostica. To isolate those fibrin degradation products capable of potentiating t-PA–mediated fibrinogenolysis, lysates were subjected to affinity chromatography at 23°C on columns containing Glu-plasminogen or t-PA coupled to CH-Sepharose 4B, and the bound fractions were eluted with TBS containing 0.05 M lysine, or with 0.4 M NaCl buffered to pH 4.0 with 0.1 M ammonium acetate, respectively, as described previously (7). The (DD)E complex was isolated by gel filtration of the material eluted from plasminogen-Sepharose on a column of Sephacryl S-300 HR (Pharmacia, Inc., Piscataway, NJ), and was characterized by PAGE and immunoblot analysis as we have described (7).

Effect of plasmin inhibitors on t-PA–induced fibrin(ogen)olysis

^{125}I-labeled fibrin clots, crude clot lysates, material that bound to plasminogen-Sepharose or to t-PA-Sepharose, or (DD)E complexes were incubated for 60 min at 37°C with citrated or heparinized plasma containing 1 μg/ml t-PA in the presence or absence of varying concentrations of aprotinin (1–200 kallikrein inhibitor units, KIU/ml) or supplemental α_2-antiplasmin (0.5–1.0 μM). The extent of t-PA–induced fibrinogenolysis and α_2-antiplasmin consumption was determined by measuring plasma levels of Bβ1-42 and α_2-antiplasmin, respectively. At intervals, 100-μl aliquots of plasma were removed, and further t-PA–induced proteolysis was terminated by the addition of D-phenyl-L-prolyl-L-arginyl chloromethyl ketone (Calbiochem-Behring Corp., San Diego, CA) at a final concentration of 10 μM. Half of each sample was used for assay of α_2-antiplasmin, while the fibrinogen in the remaining aliquot was precipitated with 150 μl of chilled ethanol followed by centrifugation at 15,000 *g* for 5 min. The ethanol supernatants were then evaporated to dryness using a Speed-vac concentrator (Savant Instruments Inc., Hicksville, NY), reconstituted to original volume with distilled water, and assayed for Bβ1-42.

The effect of human α_2-antiplasmin on t-PA–induced lysis of ^{125}I-labeled rabbit clots was examined by incubating the clots for 60 min at 37°C with heparinized rabbit plasma containing 1 μg/ml t-PA in the presence or absence of varying concentrations of human α_2-antiplasmin (0.5–1.0 μM). The extent of t-PA–induced fibrinogenolysis and α_2-antiplasmin consumption was determined by measuring plasma levels of fibrinogen and α_2-antiplasmin, respectively. At intervals, 100-μl aliquots of plasma were removed, and after further t-PA–induced proteolysis was blocked by the addition of aprotinin (500 KIU/ml), the samples were assayed for fibrinogen and α_2-antiplasmin.

The effect of plasmin inhibitors on t-PA–induced clot lysis was determined in two ways. First, the time-course of release of ^{125}I-labeled fibrin degradation products was monitored by removing 20-μl aliquots of plasma at intervals, and counting them for radioactivity. Second, the clots were removed at the end of the incubation period, and after washing three times with 500-μl aliquots of TBS, their residual radioactivity was counted for 1 min. To calculate the extent of clot lysis, the difference between the radioactivity originally incorporated into the clot and the residual radioactivity was expressed as a percentage of the original radioactivity.

Effect of α_2-antiplasmin supplementation in a rabbit model of t-PA–induced thrombolysis

Preformed ^{125}I-labeled thrombi of standard size were produced in the right external jugular veins of male New Zealand white rabbits (3–4 kg) and the effects of α_2-antiplasmin supplementation on t-PA–induced thrombolysis and α_2-antiplasmin and fibrinogen consumption were determined. In addition, this model also was used to examine the effect of α_2-antiplasmin supplementation on bleeding after t-PA–induced thrombolysis. The details of the procedures are given below.

INDUCTION OF ANESTHESIA

Anesthesia was induced by the intramuscular injection of ketamine (50 mg/kg) and xylazine (2 mg/kg) and maintained with a mixture of oxygen (1 liter/min) and 2.5% isoflurane delivered by face mask. Using 21-gauge needles fitted to no. 90 polyethylene tubing, the left femoral artery and vein were then cannulated for continuous blood pressure determination and drug delivery, respectively.

PREPARATION OF ^{125}I-LABELED JUGULAR VEIN THROMBI

After exposing the right jugular vein through a ventral skin incision, a 2 cm venous segment was identified. Using a 4-Fr Fogarty catheter introduced through the facial vein, the isolated segment was denuded of endothelium by 30 passages of the inflated balloon. Blood flow through the venous segment was then occluded by tourniquets placed proximally, distally, and around the side branches. The Fogarty catheter was withdrawn, and replaced with a cannula through which the venous segment was briefly flushed with 200 μl of a 1,000 U/ml solution of bovine thrombin (Parke-Davis and Co., Ltd., Detroit, MI), and then immediately injected with a 200-μl aliquot of homologous rabbit blood containing ^{125}I-labeled human fibrinogen (~ 200,000 cpm/ml). These maneuvers resulted in rapid clot formation within the isolated vein segment. In parallel, an additional 200-μl aliquot of rabbit blood containing ^{125}I-labeled fibrinogen was counted for radioactivity for 1 min to determine the total radioactivity incorporated into the resultant thrombus. This provides an accurate estimate of the radioactivity of the clot because pilot studies demonstrated that virtually all of the labeled fibrinogen could be recovered in the thrombus. Finally, after allowing the thrombus to age for 30 min, blood flow through the jugular vein was restored for 10 min by removing the tourniquets, and the rabbits were then randomized blindly into one of three treatment groups.

TREATMENT REGIMENS

Rabbits randomized to groups I and II were treated with t-PA, 1-mg/kg bolus followed by an infusion of 1 mg/kg over 30 min. The rabbits in group II also received a 1-mg/kg bolus of human α_2-antiplasmin, whereas those in group I were given an equivalent volume of sterile

saline. Finally, the rabbits randomized to group III served as controls, and were given equivalent volume of sterile saline.

END POINTS

The end points measured were thrombolysis, α_2-antiplasmin and fibrinogen consumption, the generation of fibrinogen degradation products, and blood loss from standardized incisions in the ear. These variables were quantified as follows:

Extent of thrombolysis. At 60 min (i.e., 30 min after discontinuing the t-PA infusion) the extent of thrombolysis was determined by carefully removing the ^{125}I-labeled clot from the jugular vein segment and counting the residual radioactivity. The percentage of clot lysis was then calculated by subtracting the residual radioactivity of the clot from the initial radioactivity and expressing this as a percentage of the initial radioactivity.

α_2-Antiplasmin and fibrinogen consumption, and the formation of fibrinogen degradation products. 1-ml blood samples were collected before, and 10, 20, 40, and 60 min after the 30-min t-PA or saline infusion was started. At each time point, a 450-μl aliquot of blood was added to each of two Eppendorf tubes prefilled with 50 μl of anticoagulant consisting of 3.8% trisodium citrate and 100 KIU of trasylol. After sedimentation of the red cells by centrifugation at 15,000 g for 5 min, the plasma was removed, and stored in aliquots at −70°C until assayed for α_2-antiplasmin and fibrinogen. To directly visualize the fibrinogen degradation products, plasma samples also were examined using SDS-PAGE and immunoblot analysis. The samples were diluted in an equal volume of 60 mM Tris-HCl containing 2% SDS, 5% glycerol, and 0.0001% biomophenol blue, heated at 100°C for 5 min, and then stored at −70°C until analyzed as described below.

Bleeding. At 60 min (i.e., 30 min after discontinuing the infusion of t-PA or saline), three 9-mm full-thickness incisions were made in the left ear using a no. 11 Bard-Parker scalpel blade. The pierced ear was then immediately immersed in a beaker containing 1 liter of sterile saline which was maintained at 37°C and constantly stirred using a heated stir plate and a magnetic stir bar. At 5, 10, 20, and 30 min, 15-ml aliquots of saline were removed, and the red cells were sedimented by centrifugation at 2,000 *g* for 10 min at 23°C. After aspirating the supernatant, the red cell pellet was resuspended in 1 ml of saline, and 10 μl of zapaglobin (Coulter Electronics, Inc., Hialiah, FL) was added to lyse the cells and to convert the released hemoglobin to cyanmethemoglobin. The absorbance of these samples was then measured at 540 nm to quantify the hemoglobin content, and by comparing these values with those of known quantities of rabbit blood treated in the same fashion, the volume of blood lost at each time point was estimated.

ASSAYS FOR FIBRINOGEN, α_2-ANTIPLASMIN, AND Bβ1-42

The fibrinogen concentrations were measured by the von Clauss method (23), whereas plasma levels of α_2-antiplasmin were determined chromogenically (24) using the synthetic substrate D-norleucyl-L-β-cyclohexylalanyl-L-arginine-paranitroanalide (Diagnostica Stago). The human fibrinogen-derived fragment Bβ1-42 was measured by radioimmunoassay using a specific antibody that does not cross-react with fibrinopeptide B or Bβ15-42 (25).

SDS-PAGE AND IMMUNOBLOT ANALYSIS OF FIBRINOGEN DEGRADATION PRODUCTS

Samples were applied to a 4–15% polyacrylamide slab gel containing 0.1% SDS, and electrophoresis was performed using a modified Laemmli discontinuous buffer system (26) under nonreducing conditions. The separated proteins were then electrophoretically transferred onto nitrocellulose membranes. After blocking in 5% (wt/vol) fat-free milk diluted in 0.15 M NaCl, the membranes were washed and incubated for 90 min with a 1:1,000 dilution of sheep anti-rabbit fibrinogen IgG (Cappel Laboratories, West Chester, PA). The washed membranes were then incubated for 60 min with a 1:1,000 dilution of alkaline phosphatase-conjugated donkey anti-sheep IgG (Sigma Chemical Co., St. Louis, MO), and developed with 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine and *p*-nitroblue tetrazolium chloride (Bio-Rad Laboratories, Richmond, CA).

DATA ANALYSIS

The data were expressed as the mean±SEM. Statistical analysis was performed using one-way ANOVA (27). The blood loss data were log-transformed to accommodate the increased variation with higher values (27).

Turbidometric assessment of the effect of fragment X incorporation on t-PA–induced clot lysis times

Fibrinogen and fragment X were each suspended in TBS containing 6 mg/ml polyethylene glycol at concentrations of 3.2 and 1.1 μM, respectively. The fibrinogen was diluted with varying amounts of fragment X or with an equivalent volume of buffer, and Glu-plasminogen was then added to each mixture to achieve a final concentration of 0.7 μM. Aliquots (244 μl each) of these mixtures were added to separated, 2-μl aliquots of thrombin, t-PA, and $CaCl_2$ placed in the bottom of microtiter plate wells. The final volume of the solution was 250 μl, whereas the final concentrations of thrombin, t-PA, and calcium were 5 nM, 0.06 nM, and 8 μM, respectively. The fibrinogen was diluted with fragment X or buffer to final concentrations ranging from 2.4 to 1.2 μM, while the fragment X concentration varied from 0.04 to 0.8 μM. The time course of fibrin clot formation and subsequent fibrinolysis was monitored continuously at 37°C using a thermomax microplate reader (Molecular Devices Corp., Menlo Park, CA) set at 405 nm. Clot formation typically occurred within 8 min as indicated by a plateau in turbidity. With subsequent fibrinolysis there was a progressive decrease in turbidity back to baseline. In each sample, clot formation was evaluated by measuring the time required for the turbidity to increase to the midpoint between baseline and plateau values, while the time required for the turbidity to decrease to the midpoint between plateau and baseline values was used to evaluate clot lysis.

Results

Effect of α_2-antiplasmin supplementation on t-PA–induced fibrin(ogen)olysis

The time-course of clot lysis was monitored by measuring the release of ^{125}I-labeled fibrin degradation products (Fig. 1 *A*), while the extent of fibrinogenolysis and α_2-antiplasmin depletion were followed by quantifying plasma levels of Bβ1-42 (Fig. 1 *B*) and α_2-antiplasmin (Fig. 1 *C*), respectively. With on-going clot lysis, there is progressive generation of Bβ1-42, and depletion of α_2-antiplasmin. Whereas supplementing the plasma with 0.5 or 1.0 μM α_2-antiplasmin inhibits t-PA–induced Bβ1-42 generation by 53 and 81%, respectively (Fig. 1 *B*), these concentrations of inhibitor have little effect on the release of ^{125}I-labeled fibrin degradation products (Fig. 1 *A*). The addition of 0.5 or 1.0 μM α_2-antiplasmin increases the α_2-antiplasmin level 1.4- and 1.9-fold, respectively, and attenuates the time-dependent decrease in α_2-antiplasmin concentrations (Fig. 1 *C*). Similar results were obtained when the experiments were repeated in heparinized plasma (data not shown) thereby indicating that α_2-antiplasmin supplementation has little effect on t-PA–induced thrombolysis even when calcium is available to allow activated factor XIII to cross-link the α_2-antiplasmin into the fibrin clot (28).

Effect of aprotinin on t-PA–induced fibrin(ogen)olysis

In contrast to α_2-antiplasmin, aprotinin produces concentration-dependent inhibition of both t-PA–induced fibrinogenolysis and clot lysis (Fig. 2). Although at lower concentrations

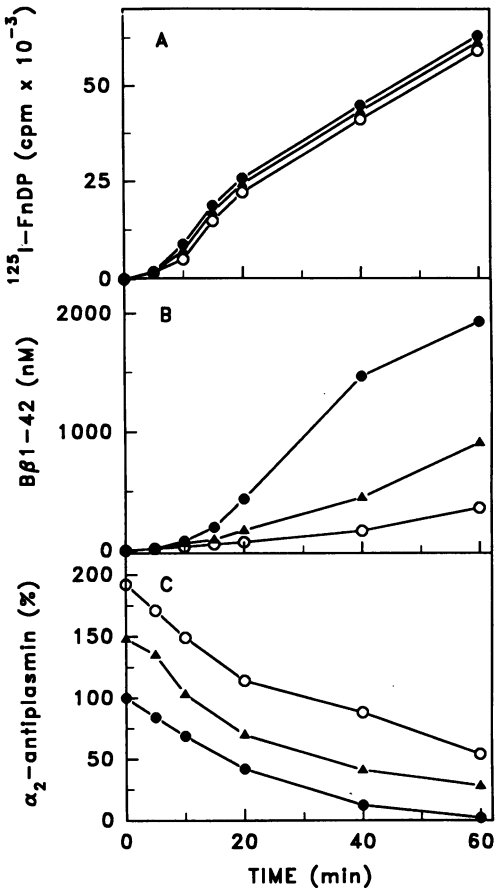

Figure 1. The effect of α_2-antiplasmin supplementation on t-PA–induced fibrin(ogen)olysis. ^{125}I-labeled fibrin clots were incubated in plasma containing 1 μg/ml t-PA for 60 min at 37°C in the absence (●) or presence of 0.5 μM (▲) or 1.0 μM (○) supplemental α_2-antiplasmin. At the times indicated, aliquots were removed and the time courses of (*A*) release of ^{125}I-labeled fibrin degradation products (^{125}*I-FnDP*), (*B*) generation of Bβ1-42, and (*C*) α_2-antiplasmin consumption were determined. Each point represents the mean of three experiments, each done in duplicate.

aprotinin blocks Bβ1-42 generation to a slightly greater extent than clot lysis, this difference is less marked when aprotinin is used in concentrations that inhibit fibrinogenolysis by 50% or more.

Effect of α_2-antiplasmin supplementation on the potentiation of t-PA–mediated fibrinogenolysis produced by clot lysates or (DD)E complex

Incubation of clot lysates (Fig. 3) or (DD)E complexes (data not shown) with citrated plasma containing 1 μg/ml t-PA markedly potentiates both Bβ1-42 generation (Fig. 3 *A*), and α_2-antiplasmin consumption (Fig. 3 *B*). Supplementing the plasma with 1 μM α_2-antiplasmin blocks the increase in Bβ1-42 levels produced by the clot lysates and (DD)E complexes by 94 (Fig. 3 *A*) and 90% (data not shown), respectively.

Comparison of the effects of α_2-antiplasmin supplementation on t-PA–induced fibrin(ogen)olysis in a human and rabbit system

To determine whether human α_2-antiplasmin has the same selective effect on t-PA–induced fibrinogenolysis in a rabbit

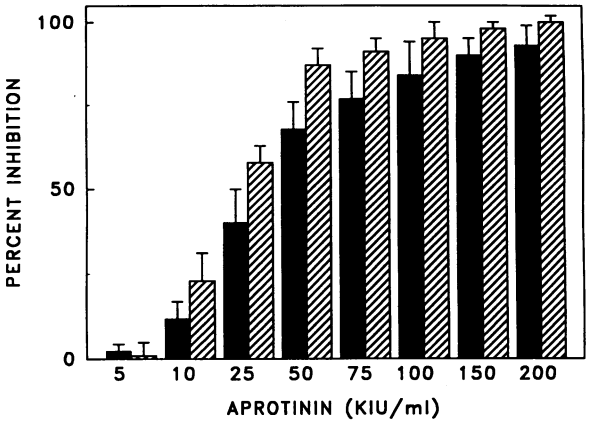

Figure 2. Comparison of the inhibitory effect of aprotinin on t-PA–induced clot lysis (*solid bars*) and Bβ1-42 generation (*hatched bars*). ^{125}I-labeled plasma clots were incubated in citrated plasma containing 1 μg/ml of t-PA for 60 min at 37°C in the absence or presence of aprotinin at the concentrations indicated. At the end of the incubation period, the extent of clot lysis was determined by counting the residual radioactivity of the clots, while Bβ1-42 levels were measured as an index of fibrinogenolysis. The percent inhibition of clot lysis and Bβ1-42 generation produced by each concentration of aprotinin was then calculated. Each bar represents the mean of three separate experiments (each done in duplicate), while the lines above the bars represent the SD.

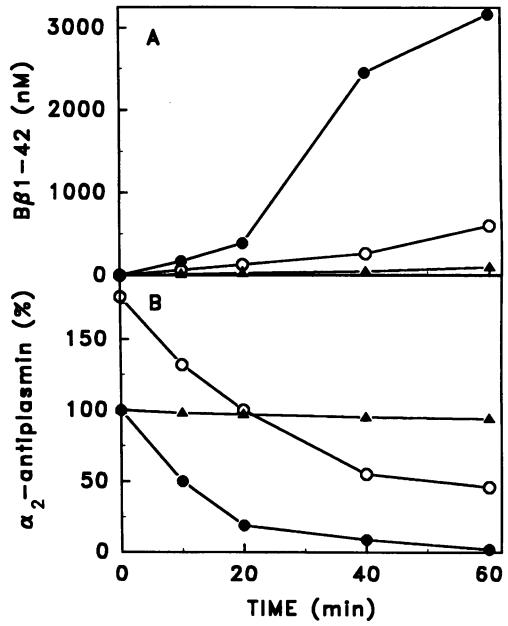

Figure 3. The effect of α_2-antiplasmin supplementation on the potentiation of t-PA–induced fibrinogenolysis and α_2-antiplasmin consumption produced by clot lysates. 35 μg of that portion of a lysate of cross-linked fibrin clots that binds to plasminogen-Sepharose was incubated with 1 ml of plasma containing 1 μg/ml t-PA for 60 min at 37°C in the absence (●) or presence (○) of 1 μM supplemental α_2-antiplasmin. At the times indicated, aliquots were removed and the time courses of (*A*) Bβ1-42 generation, and (*B*) α_2-antiplasmin consumption were determined. In a control experiment, 1 μg/ml of t-PA was incubated with plasma in the absence of the lysate (▲). Each point represents the mean of two experiments, each done in duplicate.

system as it does in human plasma, ^{125}I-labeled human or rabbit fibrin clots were incubated for 60 min at 37°C with heparinized human or rabbit plasma, respectively in the presence or absence of varying concentrations of human α_2-antiplasmin and the effect of the inhibitor on fibrinogenolysis and thrombolysis was determined. As illustrated in Fig. 4, in both the human (Fig. 4 *A*) and the rabbit system (Fig. 4 *B*), the inhibitory effect of human α_2-antiplasmin on t-PA–induced fibrinogenolysis is considerably greater than its effect on thrombolysis. These findings support the concept that the human inhibitor is suitable for use in a rabbit model of t-PA–induced thrombolysis.

Effects of α_2-antiplasmin supplementation in an in vivo model of t-PA–induced thrombolysis and bleeding

A total of 19 animals was entered in the study; 6 received t-PA alone, 7 were given the combination of t-PA and α_2-antiplasmin, and 6 served as saline controls.

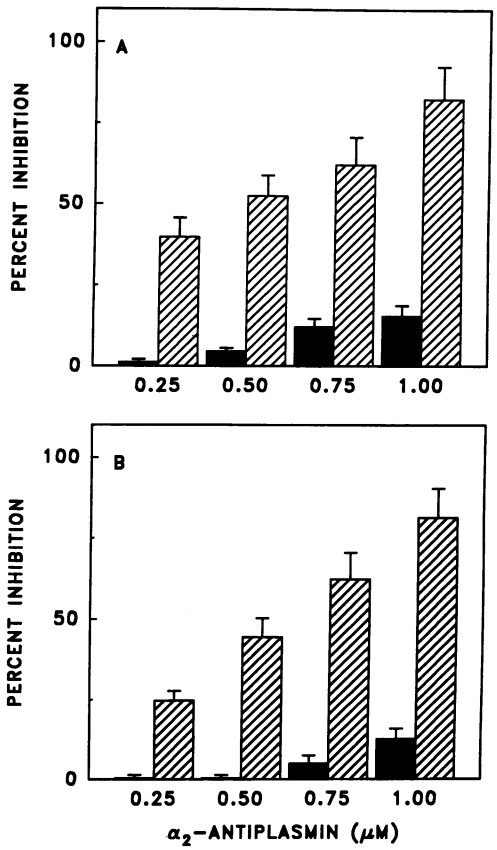

Figure 4. Comparison of the inhibitory effect of human α_2-antiplasmin on t-PA–induced clot lysis (*solid bars*) and fibrinogenolysis (*hatched bars*) in a human (*A*) or rabbit (*B*) model system, ^{125}I-labeled human or rabbit plasma clots were incubated in heparinized human or rabbit plasma containing 1 μg/ml t-PA for 60 min at 37°C in the presence or absence of supplemental α_2-antiplasmin at the concentrations indicated. At the end of the incubation period, the extent of clot lysis was determined by counting the residual radioactivity of the clots, while fibrinogen levels were measured to quantify the degree of fibrinogen consumption. The percent inhibition of clot lysis and fibrinogenolysis was then calculated for each concentration of supplemental α_2-antiplasmin. Each bar represents the mean of two experiments (each done in duplicate), while the lines above the bars reflect the SD.

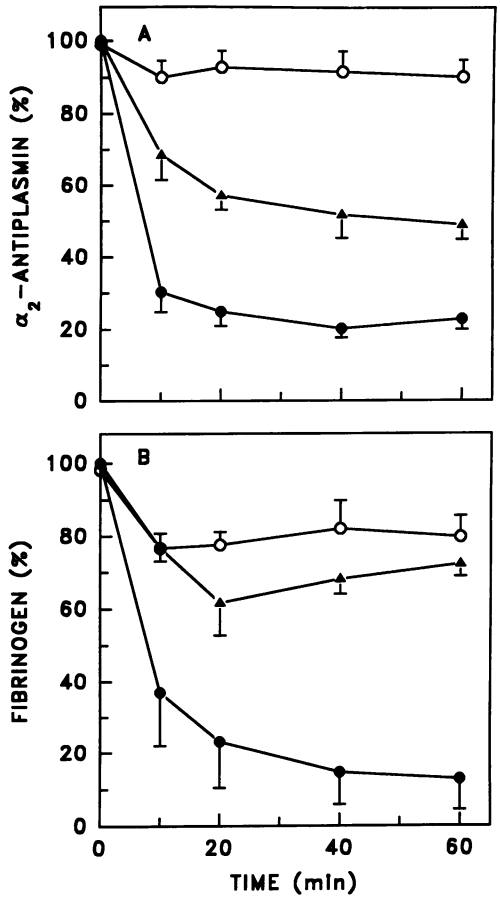

Figure 5. The effect of α_2-antiplasmin supplementation on t-PA–induced α_2-antiplasmin (*A*) and fibrinogen consumption (*B*). Rabbits were randomized to treatment with t-PA (●), the combination of t-PA and α_2-antiplasmin (▲), or saline (○), and the levels of α_2-antiplasmin and fibrinogen were measured at the times indicated. Each point represents the mean level expressed as a percentage of the pretreatment values, while the bars reflect the SEM.

Effect of α_2-antiplasmin supplementation on t-PA–induced thrombolysis. t-PA infusion produced 40.2±6.5% thrombolysis, whereas there was 5.4±1.7% clot lysis in the rabbits treated with saline ($P < 0.001$). Supplementation with α_2-antiplasmin modestly decreased the extent of thrombolysis from 40.2±6.5 to 30.1±4.0%, a difference that is not significant ($P = 0.12$). Furthermore, the extent of thrombolysis in rabbits treated with the combination of t-PA and α_2-antiplasmin was significantly greater than that in the saline-treated controls (30.1±4 and 5.4±4.6%, respectively; $P < 0.001$).

Effect of α_2-antiplasmin supplementation on α_2-antiplasmin and fibrinogen consumption. In the rabbits treated with saline, there was a 9% decrease in the mean plasma level of α_2-antiplasmin at 60 min and an 18.8% fall in the mean fibrinogen concentration (Fig. 5). The decline in the α_2-antiplasmin and fibrinogen levels in the control animals may reflect a combination of the systemic effects of a venous thrombus, and hemodilution as a result of the saline infusions. Compared to the saline-treated animals, the mean plasma levels of α_2-antiplasmin and fibrinogen were significantly ($P < 0.001$) lower in the rabbits treated with t-PA at all time points. Thus, in the t-PA–treated animals, there was a 77.3% reduction in the level

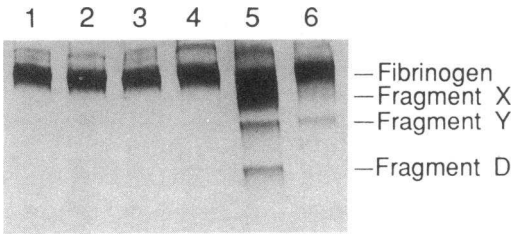

Figure 6. Effect of α_2-antiplasmin supplementation on fibrinogen proteolysis as determined by SDS-PAGE and immunoblot analysis. Representative plasma samples taken before (lanes *1–3*) and 60 min after treatment was initiated (lanes *4–6*) were analyzed in rabbits given saline (lanes *1* and *4*), t-PA alone (lanes *2* and *5*), or the combination of t-PA and α_2-antiplasmin (lanes *3* and *6*).

of α_2-antiplasmin at 60 min and an 87.3% fall in the fibrinogen concentration. By maintaining the α_2-antiplasmin levels above 45%, fibrinogen consumption is markedly reduced at all time points in the animals treated with the combination of α_2-antiplasmin and t-PA compared to that in rabbits given t-PA alone ($P < 0.001$). Furthermore, the decrease in the fibrinogen levels in the rabbits given supplemental α_2-antiplasmin is not significantly different from that in the saline controls.

To characterize the fibrinogen degradation products, plasma samples also were examined using SDS-PAGE and immunoblot analysis (Fig. 6). Whereas virtually no fibrinogen proteolysis occurs in the animals treated with saline, the administration of t-PA produces marked fibrinogen breakdown with the formation of large amounts of fragment X, as well as fragments Y and D. In contrast, much less fibrinogen proteolysis occurs when α_2-antiplasmin is given in conjunction with t-PA.

Effect of α_2-antiplasmin supplementation on bleeding. As shown in Fig. 7, α_2-antiplasmin supplementation also significantly reduces t-PA–induced bleeding. Thus, the mean cumulative blood loss over 30 min is 5,593.8±3,795.8 μl in the rabbits treated with t-PA, compared to 319.3±160 μl in the saline-treated controls ($P < 0.0001$). In contrast, the mean cumulative blood loss at 30 min in the animals given the combination of α_2-antiplasmin and t-PA is 655.9±302.2 μl, which is not significantly ($P = 0.29$) different from that in the control animals.

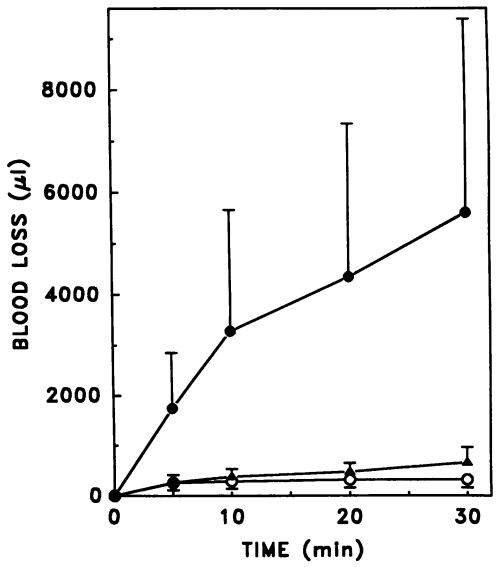

Figure 7. The effect of α_2-antiplasmin supplementation on t-PA induced bleeding. Blood loss from standardized ear incisions was measured over time in rabbits treated with t-PA (●), the combination of t-PA and α_2-antiplasmin (▲), or with saline (○). Each point represents the mean, while the bars reflect the SEM.

Effect of fragment X incorporation on clot lysis times

To explore the mechanism by which α_2-antiplasmin supplementation limits t-PA–induced bleeding, we examined the effect of fragment X incorporation on t-PA–induced clot lysis because the large amounts of fragment X which were formed in the rabbits treated with t-PA were not seen in the animals given the combination of α_2-antiplasmin and t-PA (Fig. 6). As illustrated in Table I, when fragment X is added to a solution of fibrinogen and plasminogen clotted with thrombin and $CaCl_2$ in the presence of t-PA, it shortens the lysis times in a concentration-dependent fashion. In contrast, the addition of fragment X does not influence the clotting times (data not shown) which suggests that its effects are not due to impaired polymerization of the fibrin. Further, the effect of fragment X on the lysis times is not the result of dilution of the fibrinogen because the addition of equivalent volumes of buffer in place of fragment X has no systematic effect on the lysis times.

As illustrated in Table I, the effect of fragment X on the lysis time is half maximal at a concentration of approximately 0.17 μM and is saturated at 0.4 μM fragment X. Under these conditions, the molar ratio of fibrinogen relative to fragment X ranges from 11:1 to 3:1. Thus, these findings indicate that clots formed in the presence of even small amounts of fragment X relative to fibrinogen are more susceptible to t-PA–induced lysis.

Discussion

In this study, we examined the effect of α_2-antiplasmin supplementation on t-PA–induced clot lysis, fibrinogenolysis, and

Table I. Effect of Fragment X on t-PA–mediated Clot Lysis

Fragment X	Lysis time
μM	*min*
0	73.0
0.04	69.0
0.08	58.8
0.17	51.5
0.25	43.3
0.41	36.9
0.82	37.3

Solutions of fibrinogen were diluted with increasing concentrations of fragment X and glu-plasminogen was then added. After adding aliquots of these mixtures to small separated aliquots of thrombin, t-PA and $CaCl_2$ placed in the bottom of microtiter plate wells, the plate was incubated at 37°C and the absorbance at 405 nm was measured continuously. The time required for the turbidity to decrease to the midpoint between plateau and baseline values was considered the lysis time. Each lysis time represents the mean of two experiments, each done in duplicate.

bleeding. In agreement with the findings of Paques et al. (29), our in vitro experiments demonstrate that α_2-antiplasmin supplementation inhibits t-PA–induced fibrinogenolysis but has little effect on clot lysis. However, we have extended their observations in a number of ways. First, we have shown that, like its effect in vitro, α_2-antiplasmin supplementation also blocks t-PA–induced fibrinogenolysis in vivo without any major effects on clot lysis. Second, our studies demonstrate that by inhibiting fibrinogenolysis, α_2-antiplasmin supplementation decreases t-PA mediated bleeding from standardized ear incisions. Third, our findings suggest a novel mechanism by which fibrinogenolysis can contribute to t-PA–induced bleeding.

In the in vitro studies, the extent of t-PA–induced clot lysis was determined both by measuring release of ^{125}I-labeled fibrin degradation products, and by counting the residual radioactivity of the clots, while plasma levels of α_2-antiplasmin and Bβ1-42 and/or fibrinogen were used as indices of the plasma lytic state. Since Bβ1-42 is a specific product of plasmin action on fibrinogen (25), the plasma levels of this fibrinopeptide reflect the extent of fibrinogenolysis. Whereas α_2-antiplasmin supplementation of citrated plasma has minimal effects on t-PA induced clot lysis (Fig. 1 *A*), it inhibits Bβ1-42 generation in a concentration-dependent fashion (Fig. 1 *B*) by attenuating the decrease in α_2-antiplasmin levels (Fig. 1 *C*). These findings are consistent with the concept that unlike free plasmin which is rapidly complexed and inactivated by α_2-antiplasmin, plasmin generated on the fibrin surface is relatively protected from inhibition (10–12). Protection occurs because the reaction between α_2-antiplasmin and plasmin is a two-step process (30). In the first step, a reversible complex is formed between lysine binding sites (i.e., the substrate recognition sites) on plasmin and complementary sites on the carboxy-terminal region of α_2-antiplasmin, while the second step involves irreversible inhibition of the active serine center of plasmin (12, 30). Because the lysine binding sites are inaccessible when plasmin is bound to fibrin, the clot-bound enzyme is relatively protected from inactivation by α_2-antiplasmin (10–12).

Aprotinin was used to test the validity of this explanation for our findings. This low molecular weight active-site inhibitor (31) does not interact with the substrate recognition site of plasmin, and therefore would be expected to inhibit fibrin-bound plasmin as effectively as the free enzyme. As indicated in Fig. 2, aprotinin produces similar concentration-dependent inhibition of both t-PA–induced clot lysis and Bβ1-42 generation thereby supporting this concept.

In our previous study, we demonstrated that t-PA–induced fibrinogenolysis is mediated by fibrin degradation products through their capacity to potentiate plasminogen activation by binding t-PA and plasminogen (7). Further, we showed that of the fibrin degradation products, (DD)E complex is the most efficient stimulator. The current experiments extend these observations by demonstrating that α_2-antiplasmin supplementation blocks the increase in α_2-antiplasmin consumption and Bβ1-42 generation that occurs when soluble fibrin degradation products (Fig. 3) or isolated (DD)E complexes (data not shown) are added to t-PA containing plasma. These findings suggest that unlike plasmin bound to fibrin, the plasmin generated through the potentiating effects of soluble fibrin derivatives is susceptible to inactivation by α_2-antiplasmin.

To determine the effects of α_2-antiplasmin supplementation in vivo, rabbits with radioactive jugular vein thrombi were randomly allocated to receive either t-PA, t-PA together with α_2-antiplasmin, or saline and then the extent of thrombolysis, fibrinogen proteolysis, and bleeding was determined. We were able to use human α_2-antiplasmin in these studies because in vitro experiments demonstrated that human α_2-antiplasmin selectively blocks t-PA induced fibrinogenolysis in a rabbit system to the same extent as it does in human plasma (Fig. 4).

Like its effects in vitro, α_2-antiplasmin supplementation has only a modest effect on t-PA–induced clot lysis in vivo. However, by maintaining the α_2-antiplasmin levels above 45%, the extent of fibrinogenolysis is markedly reduced (Fig. 5) such that the mean fibrinogen concentrations in the animals that received supplemental α_2-antiplasmin in addition to t-PA are not significantly different from those in the saline control group. Thus, these findings indicate that α_2-antiplasmin supplementation selectively blocks fibrinogenolysis with much less of an effect on thrombolysis both in vivo and in vitro.

To explore the link between t-PA–induced fibrinogenolysis and bleeding, we examined the effect of α_2-antiplasmin supplementation on blood loss measured 30 min after stopping the t-PA or saline infusions. As illustrated in Fig. 7, α_2-antiplasmin supplementation reduces blood loss by 94% such that bleeding in these animals is similar to that in the saline controls.

The mechanism of t-PA–induced bleeding is controversial. The currently accepted explanation, which is probably correct, is that the major mechanism by which t-PA produces hemorrhage is by degrading the fibrin within the hemostatic plug. Our findings suggest that the composition of the fibrin within the hemostatic plug influences its susceptibility to lysis and hence may also contribute to t-PA–induced bleeding. Specifically, we have shown that t-PA converts a large proportion of the fibrinogen to fragment X (Fig. 6), a finding consistent with that of Owen and colleagues (2). Since fragment X is clottable (32), it becomes incorporated into the fibrin network of the hemostatic plug and we have demonstrated that fibrin clots formed in the presence of fragment X are more susceptible to t-PA–induced lysis (Table I).

In summary, we have shown that unlike free plasmin, clot-bound plasmin is relatively protected from inactivation by α_2-antiplasmin. As a result, α_2-antiplasmin supplementation blocks t-PA–induced fibrinogenolysis with relatively little effect on thrombolysis. Fibrinogen proteolysis with resultant fragment X formation may contribute to t-PA–induced bleeding because fragment X incorporation into the fibrin matrix of newly formed hemostatic plugs renders them more susceptible to lysis. By limiting fragment X formation, α_2-antiplasmin reduces t-PA–induced bleeding in the rabbit ear model. Extrapolation of our results in rabbits to clinical bleeding with t-PA must be done with caution because the blood vessels severed in the rabbit ear are small and under low pressure. Nevertheless, our findings raise the possibility that strategies aimed at limiting fibrinogenolysis and fragment X formation may improve the safety of fibrin-specific plasminogen activators.

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